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(54) Title: P53–DEPENDENT SECRETION OF GROWTH INHIBITORY FACTORS

(57) Abstract

The present invention relates to a method for preparing a preparation of p53–dependent growth inhibitory factors for use in a pharmaceutical composition. Cultured human cells, capable of expressing p53 tumor suppressor protein, are induced to express and secrete p53–dependent growth inhibitory factors into the culture medium, where the growth inhibitory factors are recovered to prepare a preparation for use in a pharmaceutical composition. This pharmaceutical composition is then used in a method for reducing unwanted or abnormal cell proliferation by administering an effective amount to a subject in need thereof.

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P53-DEPENDENT SECRETION OF GROWTH INHIBITORY FACTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority under 35 U.S.C. §119(e) from U.S. Provisional application nos. 60/075,490, filed February 23, 1998, and 60/084,755, filed May 8, 1998, the entire contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the p53-dependent secretion of growth inhibitory factors and more particularly to a method for preparing p53-dependent growth inhibitory factors for use in a pharmaceutical composition and a method for reducing cell proliferation.

Description of the Related Art

The p53 tumor suppressor gene encodes a nuclear transcription factor (tumor suppressor protein), that accumulates in the cells in response to a variety of stresses and that is a key component of a cellular mechanism of stress response (for review see Gottlieb et al., 1996). This tumor suppressor protein, which has rapid turnover, is stabilized in the cell under various stressful conditions including DNA damage (Maltzman et al., 1984; Kastan et al., 1991), deregulation of microtubules (Tishler et al., 1995), hypoxia (Graeber et al., 1996) and detachment from its natural substrate (Nikiforov et al.). Normal function of p53 is required for control of cell aging (Atadja et al., 1995). Cell response to certain growth factors also requires p53 (Bacus et al., 1996). p53 activation, depending on cell type, results in growth arrest at one of the cell cycle check points or apoptosis (Michalovitz et al., 1990).

The biological effects of p53 are cell type specific. For example, *in vivo* it determines massive apoptosis in response to gamma irradiation or treatment with

chemotherapeutic drugs only in early embryos and several sensitive tissues, including spleen and thymus (Lowe et al., 1993a; MacCallum et al., 1996; Komarova et al., 1997). Many p53-mediated effects are achieved through the activity of p53-responsive genes.

Functional inactivation of p53 occurs in the majority of cancers and established cell lines (Vogelstein et al., 1992). It is achieved by mutations (many of which generate dominant negative variants of p53), deletions or inhibitory activity of p53-interacting proteins of cellular (such as mdm-2) or viral (such as large SV40 T-antigen, E6 protein or papilloma virus, etc.) origin (see references cited in Gottlieb et al., 1996). Lack of p53 in normal cells leads to the loss of genomic stability (Yin et al., 1992), spontaneous immortalization (Donehower et al., 1992), abrogation of apoptosis (Clarke et al., 1993 and Lowe et al., 1993a) and as a result, susceptibility to transformation with dominant oncogenes (Olson et al., 1994). Loss of p53 by tumor cells may be associated with high resistance to chemo- and radiation therapy and the establishment of metastatic behavior through suppression of p53-mediated programmed cell death (Nikiforov et al.; Lowe et al. 1993a; Lowe et al., 1993b and Thompson et al., 1995). In normal development, p53 serves to control apoptosis elimination of genetically damaged cells in early stages of embroyogenesis (Norimura et al., 1996 and Komarova et al., 1997). Lack of p53 in p53 knock-out mice leads to a high incidence of spontaneous cancers in adult animals (Donehower et al., 1992; Jacks et al., 1994) and to high frequency of teratogenic abnormalities in embryos exposed to ionizing radiation (Norimura et al., 1996). The exact mechanisms by which p53 exerts its growth-suppressor and apoptoxic activities are poorly understood, although some of the components of the p53-medicated control pathway have been identified (Gottlieb et al., 1996). At least some of the p53-mediated effects are determined by the ability of p53 to act as a nuclear transcription factor. The activity of p53-responsive genes accounts in part for p53-mediated checkpoint

control (*waf1; 14-3-3*), apoptosis (*bax*), genes determining reactive oxygen metabolism, and p53 feedback regulation (*mdm2*) (El-Deiry et al.,1993; Hermeking et al., 1997; Kastan et al., 1992; Wu et al., 1993; Miyashita et al., 1995; Okamoro et al., 1994; Buckbinder 1995; Polyak et al., 1997; Momand et al., 1992 and Kondratov et al., 1995). In addition, p53 can suppress transcription from a number of promoters (Mack et al., 1993) and directly inhibit DNA replication (Cox et al., 1995).

Anti-cancer therapy regimens involve a variety of stress-inducing treatments (radiation, chemotherapeutic drugs, hyperthermia, etc.) the toxicity of which is higher for cancer cells than for normal cells. Many of these treatments lead to the activation of p53 and subsequent p53-dependent growth arrest or apotosis (Gottlieb et al., 1996). The conventional mode of thinking is that cell death occurs as a result of damage of intracellular components that trigger cell death, and the anti-cancer effect is believed to be associated either with stronger damage or with stronger apoptotic response of malignant versus normal cells. Since anti-cancer therapy is usually damaging not only to the tumor but also to normal tissues, much effort has been made in targeting stress treatment specifically and selectively at cancer cells. However, there is a bulk of evidence indicating that anti-cancer therapy with stress-inducing treatments is not limited to the internal damage occurring within cancer cells after therapeutic treatment. For example, shielding the tumor in a tumor-bearing animal does not completely prevent it from exhibiting the anti-tumor effect of whole-body gamma irradiation (Seung et al., 1996). A decreased ability of tumors to grow in irradiated tissues, known as the "tumor bed effect", is a well-known phenomenon (Milas et al., 1988; O'Brien et al., 1969; Urano 1967).

In response to treatment with radiation or hyperthermia, or with DNA-damaging or microtubule-targeted chemotherapeutic drugs, p53 protein accumulates in the cell

(Gottlieb et al., 1996). The tolerable doses of anticancer drugs and radiation are limited by their toxicity to several sensitive tissues, including hematopoietic organs, where transgenic animal studies showed that this effect is p53 dependent (Komarova et al., 1997). Thus, the efficacies of chemo- and radiation therapies are mediated by p53, and at the same time, are limited by p53-dependent toxicity for normal tissues.

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SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the deficiencies of the prior art.

The present invention firstly provides a method for preparing a preparation of p53-dependent growth inhibitory factors from a culture of normal human cells in a nutrient medium for use as antiproliferative agents, such as an anti-cancer therapeutic.

The present invention further provides a pharmaceutical composition comprising a preparation of p53-dependent growth inhibitory factors prepared from a culture of human cells induced to express and secrete p53-dependent growth inhibitory factors.

Also provided by the present invention is a method for reducing unwanted or abnormal cell proliferation by administering a pharmaceutical composition containing a preparation of p53-dependent growth inhibitory factors.

Another object provided by the present invention is a method for preventing or delaying cell senescence by administering a pharmaceutical composition containing an agent which attenuates the secretion of or neutralizes the growth

suppressive effects of one or more p53-dependent growth inhibitory factors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a graph of the results of a tumor growth inhibition assay, which demonstrates tumor suppression *in vivo* by conditioned medium. Mice were examined weekly for tumor growth. Four weeks after injection, tumors developed in approximately half of the injected sites in the control group, while the experimental group of injected animal tumors appeared in only three of 20 sites injected. This result suggests that factors secreted by irradiated splenocytes possess *in vivo* detectable anticancer activity.

Figures 2A and 2B show detection of p53-responsive genes by differential hybridization with ATLAS cDNA Array (CLONTECH Laboratories, Inc., Palo Alto, CA) (Fig. 2A) and Northern blot hybridization (Fig. 2B). In Fig. 2A, similar areas of nylon membranes hybridized with ^{32}P-labeled cDNA from three organs (liver, spleen, and thymus) of untreated and gamma-irradiated p53-null and wild type mice are shown. Radiation responses of three indicated serine protease inhibitors illustrate the tissue-specificity and p53-dependence of their expression. The results of hybridization to cDNA arrays were confirmed by Northern blot hybridization for serine protease inhibitor 2 (Fig. 2B). The abbreviations shown in Figs. 2A and 2B are as follows: 1 - α1-protease inhibitor 2; 2- serine protease inhibitor 2; 3- serine proteinase inhibitor 2.4; -wt- wild type; -/- -p53 knockout; u/t - untreated; and γ - gamma irradiated.

Figures 3A and 3B show different patterns of p53-dependent gene regulation as determined by Northern hybridization with RNA from cells that have been treated as indicated - untreated (u/t) MDAHO41 cells gamma irradiated (γ) MDAHO41 cells, tetracycline added (+ tetr) TR9-7 cells, no tetracycline added (- tetr) cells and gamma irradiation + tetracycline added (γ + tetr).

Figures 4A-4F show that growth inhibition of prostate tumor cells DU145 by conditioned media and urine depends on p53 activation and p53 induction (Figs. 4A-4C) and p53 induction by Western immunoblotting (Figs. 4D-4F). Bar diagrams (Figs. 4A-4C) show cell growth rate estimated as percent of ^{3}H-thymidine incorporation by the DU145 cells, growing in the presence of conditioned medium from untreated (left bar of bar pairs, considered as 100%) and gamma-irradiated (right bar of bar pairs) cells. Cells used as sources of conditioned media: Fig 4A - mouse embryo fibroblasts (MEF) transformed with Ela + ras, p53-deficient (-/-), p53 wild type (wt), transduced with either bcl-2 (bcl-2) or anti-p53 GSE (GSE); Fig. 4B - Balb 3T3 cells, line ConA (wt) and their derivatives expressing anti-p53 GSE (GSE); and Fig. 4C - human skin fibroblasts MDAH041 (-/-) and TR9-7 (wt). The hatched bar in Fig. 4C corresponds to human fibroblasts (line TR9-7) with tetracycline-regulated wild type p53 activated by tetracycline deprivation. Figs. 4D-4F show the results of Western immunoblotting with MEF (Fig. 4D) and human fibroblasts (Fig. 4F), demonstrating p53 induction after gamma irradiation, and X-gal staining of ConA cells (marked Balb 3T3) (Fig. 4E).

Figures 5A-5C shows the growth suppressive effect of conditioned media from short-term cultures of irradiated p53-null (p53 -/-) and wild type (wt) splenocytes (Fig. 5A) collected at the different time points indicated and urine (Fig. 5B) from p53-null and p53-wild type mice before (control) and after gamma irradiation (gamma). Immunoperoxidase staining of irradiated p53-wild type and p53-null splenocytes from p53 is shown in the left bottom corner of Fig. 5A. In the representative experiment shown, urine samples from six p53-wild type (crosses) and six p53-null mice (dots) harvested before and after irradiation were tested individually (Fig. 5B). Fig. 5C shows a comparison of growth suppressive effect of different dilutions of conditioned medium from gamma irradiated ConA cells on different indicated target cells (NMuMG, HSF, and DU145). The conditioned serum-

free medium was concentrated (eight fold) by ultrafiltration through Amicon filter with 10 kd cutoff.

Figures 6A-6D show adriamycin induced degradation of colonies of multidrug resistant KB/8-5-11 cells growing on p53-wild type (Figs. 6A and 6C), but not on p53-null (Figs. 6B and 6D), human fibroblasts. Representative microscopic view at 10x (Figs. 6A and 6B) and 40x (Figs. 6C and 6D) magnification of colonies of KB8-5-11 cells after 5 days of treatment with 50 ng/ml of adriamycin are shown.

Figure 7 shows a graph of a comparison of adriamycin dependence on numbers of colonies formed by KB8-511 cells plated on wild type and p53-deficient human fibroblast.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery by the present inventors that the activation of p53 in normal noncancerous cells induces the expression and secretion of growth inhibitory factors possessing anti-cancer activity. The present inventors found an accumulation of radiation-induced factors, which cause antiproliferative effects on a variety of tumor cell lines, in the media from established and primary cell cultures and *in vivo* in the urine of mice after gamma irradiation *in vitro*. Moreover, it was found that p53-dependent factors released by normal cells potentiated the cytotoxic effect of chemotherapeutic drugs for co-cultivated tumor cells. Accordingly, p53-dependent growth inhibitory factors secreted by normal cells play a role in the chemo- and radiation therapies for treating cancer.

One aspect of the present invention is directed to a method for preparing a preparation of p53-dependent growth inhibitory factors from cultured normal human cells by subjecting the cell culture to a stress treatment, i.e., gamma irradiation, to induce the expression and secretion of p53-dependent growth inhibitory factors. The p53-dependent growth inhibitory factors secreted into the culture medium to form a conditioned medium is then recovered to prepare a preparation of p53-dependent growth inhibitory factors for use in the

second aspect of the invention, which is a pharmaceutical composition for reducing unwanted or abnormal cell proliferation.

This method for preparing a preparation of p53-dependent growth inhibitory factors involves culturing human cells so that p53 tumor suppressor protein is expressed in response to a stress treatment (i.e., preferably gamma irradiation), which p53 tumor suppressor protein acts to induce the expression and secretion of p53-dependent growth inhibitory factors into the culture medium. The conditioned medium formed by the release of growth inhibitory factors into the nutrient culture medium is then processed to recover the p53-dependent growth inhibitory factors (i.e., TGF-beta 2, inhibin-beta, and serine protease inhibitors) and provide a preparation of such growth inhibitory factors.

The human cells used in the method according to the present invention are preferably fibroblasts cells, spleen or thymus cells, and mixtures thereof. Other suitable cells for use in the present method can be readily identified by determining whether they express p53 in response to a stress treatment, the expression which of then induces the expression and secretion of one or more p53-dependent growth inhibitory factors. As the selection of p53-dependent growth inhibitory factors expressed vary depending on cell type, a mixed culture of different cell types can be used to produce a preparation containing a broad spectrum of p53-dependent growth inhibitory factors.

Many mammalian cell cultures are routinely grown in nutrient culture media containing serum, where fetal bovine serum (FBS) is probably the most extensively used serum for mammalian cell culture, although other mammalian sera are also used. However, the use of serum poses a number of problems. Serum is an expensive commodity which is not readily available in amounts required for commercial production. It is also a biochemically undefined material. Serum is known to contain many major components including albumin and transferrin and also minor components, many of which have not been fully

identified nor their action determined. Thus, serum will differ from batch to batch possibly requiring testing to determine levels of the various components and their effect on the cells. Frequently, serum is contaminated with microorganisms such as viruses and mycoplasma, many of which may be harmless but will represent an additional unknown factor. This problem has become more acute in recent years with the emergence of Bovine Spongiform Encephalopathy (BSE). Despite improvements in screening, regulatory authorities are likely to require the sourcing of bovine products from those areas which are free from (BSE) infections. Furthermore, the presence of animal proteins in culture media can require lengthy purification procedures.

While the conventional culture of human cells in a nutrient culture medium containing serum can be used in the method of the present invention, it is preferred that the culture medium used for producing a preparation of p53-dependent growth inhibitory factors be serum-free. Such serum-free media, with or without specific substitutes for components/factors in serum, is a defined media from which a preparation of p53-dependent growth inhibitory factor can be purified. Examples of such serum-free media suitable for human cells are the commonly used DMEM and RPMI media are well known. Many other serum-free media with or without substitutes for serum component are also well known in the art with some being commercially available from suppliers such as Life Technologies, Gaithersburg, MD. Those of skill in the art are furthermore quite capable of optimizing the defined components of a serum-free media for the growth of the cell type(s) to be used in the method according to the present invention.

As the serum-free media is preferably a defined media, all the components of the media are known. Therefore, one of skill in the art is quite capable of developing with only routine experimentation a purification scheme for removing media components to prepare a preparation of p53-dependent growth inhibitory factors suitable for administering

to a subject in a pharmaceutical composition. Depending on the components of the serum-free culture medium used in the method of the present invention, repeated cycles of ultrafiltration and washes may sufficiently remove the media components that little or no further purification procedures are needed to prepare the preparation of p53-dependent growth inhibitory factors.

The preparation of p53-dependent growth inhibitory factors prepared according to the method of the present invention can be formulated into a pharmaceutical composition for use in reducing unwanted or abnormal cell proliferation. An effective amount of the pharmaceutical composition is administered to a subject in need thereof, such as a cancer patient, in a method for reducing cell proliferation where the proliferation of cells is unwanted or abnormal, i.e., neoplasms and malignant cancer cells. While the method for reducing unwanted or abnormal cell proliferation may involve the administration of the pharmaceutical composition containing a preparation of p53-dependent growth inhibitory factors as the sole treatment, it may be preferable and advantageous to combine this administration with conventional chemotherapy and/or radiation therapy to potentiate the cytotoxic effects of chemotherapeutic drugs or enhance the antiproliferative effects of radiation therapy.

The pharmaceutical composition of the present invention is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

In the method for reducing unwanted or abnormal cell proliferation according to the present invention, the compound of the present invention can be administered in various ways. It should be noted that the preparation of p53-dependent growth inhibitory factors can be administered alone as the pharmaceutical composition or as active ingredients in combination with pharmaceutically acceptable carriers, diluents, excipients, adjuvants, vehicles and auxiliary agents. The pharmaceutical composition can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants containing the preparation are also useful. Moreover, it may be advantageous and preferable to administer the pharmaceutical composition according to the present invention locally at or near the site of unwanted or abnormal cell proliferation, i.e., tumors. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses over a period of several days, but single doses are preferred.

When administering the pharmaceutical composition of the present invention parenterally, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example,

water, ethanol, polyol (for example, glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such as cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, may also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the preparation of p53-dependent growth inhibitory factors utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of

delivery systems useful in the present invention include: 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

It is contemplated that a pharmacological formulation of the compound utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver it orally or intravenously and retain the biological activity are preferred.

In one embodiment, the pharmaceutical composition of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity to be administered will vary for the patient being treated and will vary from about 100 ng/kg of body weight to mg/kg of body weight per day and preferably will be from 10 μg/kg to 10 mg/kg per day.

The laboratory of the present inventors have also discovered that the onset of senescence in rat and chicken embryo fibroblasts is associated with the accumulation of growth inhibitory factors in conditioned medium. Culture media conditioned by senescent rat embryo fibroblasts (REFs) was growth suppressive to a broad variety of target cells including young REFs and a series of tumor cell lines. The growth suppressive effect was neutralized after 1 hr. incubation of conditioned media at 56°C. These observations suggest that the aging of a cell population is a cooperative process which involves growth suppression of proliferating cells by growth inhibitors released by gradually accumulating senescent cells.

Accordingly, a further aspect of the present

invention is directed to a method for preventing or delaying cell senescence, which involves administering to a subject in need thereof a pharmaceutical composition containing an agent which has the activity of directly or indirectly attenuating the secretion of or neutralizing the growth suppressive effects of p53-dependent growth inhibitory factors released by gradually accumulating senescent cells. The features of formulating the agent in a pharmaceutical composition, dosage and how it is administered, as discussed above with the pharmaceutical composition containing a preparation of p53-dependent growth inhibitory factors, also apply to a pharmaceutical composition containing this agent for attenuating the secretion of or neutralizing the growth suppressive effects of p53-dependent growth inhibitory factors.

Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration and is not intended to be limiting of the present invention.

EXAMPLE

The present inventors proposed that p53 can act as a tumor suppressor by inhibiting growth of damaged cells through internal control of cell cycle and apoptotic machinery and that the activation of p53 in normal cells induces the production and release of growth inhibitory factors possessing anti proliferative/anti-cancer activity. To test this hypothesis, the growth inhibitory effect of conditioned media from the cells differing in their p53 status before and after p53 activation was compared. Three experimental cell systems, representing pairs of isogenic cell lines differing in their p 53 status or activity, were specifically designed to analyze p53 function as follows:

1) p53-null human fibroblasts, strain MDAHO41 (Agarwal et al., 1995), originally obtained from a Li-Fraumeny syndrome patient. MDAHO41 cells were compared with their derivatives, TR9-7, obtained by introduction of wild type p53

cDNA under the control of tetracycline-inducible promoter (Agarwal et al., 1995). The level of p53 expression in TR9-7 in the presence of tetracycline is comparable to that of normal p53-containing fibroblasts. p53 activation (stabilization) in TR9-7 was achieved either by gamma irradiation in the presence of tetracycline or by deprivation of the antibiotic.

2) Balb 3T3 cells of cell line 12-1, express wild type p53 (Harvey et al., 1991). This line was compared with its variant obtained by retroviral transduction with anti-p53 GSE-56 acting as a strong dominant negative inhibitor of p53 function (Ossovskaya et al., 1996). p53 activation was achieved by gamma irradiation.

3) Mouse embryo fibroblasts (MEF) with wild type p53 transformed with a combination of Ela and transforming Ha-ras (cell line C8) were compared with their derivatives transduced with retroviral vectors expressing either anti-p53 genetic suppressor element (GSE) C2 (analog of GSE-56) or bcl-2 (Nikiforov et al., 1997). The bcl-2-expressing construct was used to prevent radiation-induced p53-dependent apoptosis in C8 cells that may affect the properties of conditioned media.

All the above cells were gamma irradiated (10 Gy) to induce p53 in response to DNA damage, and conditioned media was collected 8 hours after irradiation and compared with that from control non-irradiated cells in a growth inhibitory assay. In tet041 cells p53 was also activated with tetracycline deprivation. A set of target prostate carcinoma cell lines, ALVA, PC3, DU145, LNCAP, was used for growth inhibition studies (Rokhlin et al., 1997). The effect of conditioned media on growth of target cells was estimated by ^{3}H thymidine incorporation after 12 hours of incubation in conditioned media. As similar results were obtained for the different target cell lines and, therefore, further analysis was done using DU145 cells, known to be the most resistant among the tested cell lines to conventional anti-cancer treatments (low sensitivity to TGF-beta, interferon gamma, TNF

alpha, lack of apoptosis after treatment with adriamycin) (Rokhlin et al., 1997).

In all three cell systems, growth inhibition of target cells was evident only with the media from p53 wild type cells after p53 activation. No such effect was detected in the media from p53-negative cells regardless of whether they were irradiated or not. Conditioned media from untreated p53 wild type cells did not affect growth of target cells as well. These observations indicate that cells can release growth inhibitory factors in the media in response to stress-treatments. It is important to note that irradiation of human fibroblasts and Balb 3T3 cells did not lead to apoptosis within the time of the experiment as judged by DNA degradation assay. Moreover, bcl-2 does not prevent secretion of growth inhibitory factors into media by C8 cells, although it blocks apoptosis associated in C8 cells with p53 activation.

Secretion of growth inhibitory factors by cells with wild type p53 in response to genotoxic stress (i.e., gamma irradiation) raised the possibility that normal cells may contribute to anti-tumor effect of radiation and chemotherapy by releasing tumor-suppressive factors. This model provides a possible explanation for well known phenomenon of "tumor bed effect". To test this idea, multidrug resistant HeLa cells, line KB8-5-11, overexpressing P-glycoprotein (P-gp) was used. These cells are highly resistant to a variety of P-gp substrates, many of which are used as chemotherapeutic drugs (etoposide, adriamycin, Vinca alkaloids, etc.) (Roninson et al., 1986). KB8-5-11 cells were plated (1000 cells per plate) on sparse monolayers of either p53-deficient human fibroblasts (MDHAO41) or their derivatives expressing wild type p53 (TR9-7). When KB8-5-11 cells formed small colonies, 25mg/ml of adriamycin, which is completely growth suppressive for human fibroblasts (both lines) but does not affect growth of KB8-5-11 cells, was added. The majority of adriamycin resistant colonies on monolayer of cells with wild type p53 degraded while similar colonies on p53-deficient cells continued to grow. Morphological changes in dying cells indicated that

cell death occurred through activation of apoptosis. This observation demonstrates that tumor cells can be killed by chemotherapeutic drug indirectly through the p53-dependent induction of growth suppressive factors in normal stromal cells.

A similar experiment was done on colonies of spontaneously transformed Balb 3T3 cells (line ConA carrying lacZ gene under control of p53-responsive promoter, Roninson et al., 1986) growing on human fibroblasts differing in their p53 status (MDHAO41 and TR9-7). TR9-7 carry p53 cDNA under the control of tetracycline-regulated promoter and activation of p53 in them was achieved by tetracycline deprivation.

Several genes that are known to be p53-regulated encode secreted growth inhibitory proteins. For example, thrombospondin and IGFBP3 both are encoded by the genes that are upregulated by p53 (Buckbinder et al., 1995; Dameron et al., 1994). New candidate secreted growth inhibitors can be found among other p53-responsive genes found by different methods of identification of the genes whose expression depends on p53 status. Thus, differential hybridization of ATLAS cDNA arrays (CLONTECH Labs, Palo Alto, CA) with cDNA probes obtained from gamma-irradiated p53-positive and negative human fibroblasts and from irradiated spleens of p53-wild type and p53-deficient mice revealed several new candidate excreted growth suppressors including TGF-beta2, inhibin-beta and a set of serine protease inhibitors. Observations made by others were published suggesting the use of serine protease inhibitor expression as potential prognostic factors for the estimation of the efficacy of radiation therapy and the risk of spontaneous cancer (Benitez-Bribiesca et al, 1989 and Schelp et al., 1998). Chemical inhibitors of serine protease have been used as anti-tumor drugs for a number of years (DeClerck et al., 1994). These facts clearly fit the present findings that link anti-cancer effect of this class of anti-cancer secreted proteins with the activity of p53 and anti-cancer therapy.

The obtained results indicate that anticancer

therapy can inhibit growth of tumor cells indirectly by induction of p53-dependent secretion of growth inhibitory factors by normal cells. Such factors can be found among p53 transactivators in culture media and presumably, in blood or other fluids contacting with p53-wt cells in the organism after anti-cancer treatment p53-dependent secretion of growth inhibitory factors in response to stress is likely to explain the mechanism of tumor bed effect of local gamma radiation on proliferation of non-irradiated tissues. These factors could be involved in control of aging since senescent cells accumulated in aging tissues are known to activate p53-dependent gene expression serving as a source of growth inhibitors affecting proliferation and regeneration of tissues.

The materials and methods used in the experiments in this example are presented below along with a detailed discussion of the results obtained.

MATERIALS AND METHODS

Cell lines

p53-negative Li-Fraumeni fibroblasts (line MDAHO41) their derivative expressing wild type p53 under the control of a tetracycline-regulated promoter (line TR9-7) and Balb/c 3T3 cells 12-1 (Harvey et al., 1991) with wild type p53 expressing *IacZ* under control of p53-dependent promoter (line ConA) were described earlier (Komarova et al., 1997; Agarwal et al., 1995) and were kindly provided by George Stark. Mouse embryo fibroblasts transformed with E1a+ras from p53-wild type (line C8) and p53-null (line A4) fibroblasts were kindly provided by (Lowe et al., 1993a). The derivatives of ConA and C8 cells overexpressing bcl-2 or GSE-56 were obtained by retrovirus transduction of the above cDNAs (Nikiforov et al., 1997). Mouse breast epithelial cell line NMuMG and normal diploid skin fibroblasts were obtained from ATCC. All cell lines were cultivated in DMEM with 10% fetal calf serum. TR9-7 cells were maintained continuously in the presence of 1 μg/ml of tetracycline; to induce p53 expression, cells were incubated

without tetracycline for 24 hours. Prostate tumor cells ALVA, PC3, DU145 and LNCAP were previously described (Rokhlin et al., 1997). Short-term cultures of splenocytes and thymocytes (2-3x10^7 cells/ml) were prepared from freshly isolated and gently desegrated mouse spleens and thymus glands in RPMI media supplemented with 10% fetal calf serum.

Animals

p53-knockout and control mice (both C57BL) were purchased from Jackson labs, Bar Harbor, Maine.

Gamma-irradiation

Mice and cell cultures were irradiated (10 Gy) from a G.L. Shepherd and Associated Model 143-68 Cs137 source at a dose rate of 4 Gy per min.

cDNA array hybridization

Differential hybridization analysis was done with CLONTECH ATLAS cDNA arrays (MacCallum et al., 1996) and with cDNA microarrays provided by QBI Enterprises, Ltd. The ATLAS array consists of 588 selected cDNAs on nylon membrane (MacCallum et al., 1996). QBI arrays were prepared according to previously described technology (Schena et al., 1995; website www.clontech.com) by Synteni and contain approximately 20,000 cDNAs. Poly(A)$^+$ RNA was purified according to standard protocols from different mouse tissues and human cell lines. cDNA probe preparation and hybridization was done according to published protocols and manufacturer's recommendations (MacCallum et al., 1996; website www.clontech.com).

Preparation of media conditioned by gamma-irradiated human splenocytes

Fresh human spleen was gamma irradiated (10Gy), desegregated into a single-cell suspension in DMEM medium (5x10^7 cell/ml)

and incubated at 37°c for 7 hours. Cells were removed by centrifugation and filtration of the supernatant through 0.45 μM filter. Conditioned medium was tested in growth inhibition assay on DU145 cells.

Growth inhibition assay

Cell proliferation rate was estimated using ^{3}H-thymidine incorporation as previously describer (Rokhlin et al., 1997). The experiments were repeated three times using four parallel wells for each tested sample. Conditioned media from gamma-irradiated and control cells and urine from irradiated and control mice were collected 8 hours after irradiation, diluted at different proportions with fresh media (5-50% for conditioned media and 0.1-1% for urine) and loaded on target cells for 48 hours. ^{3}H-thymidine was added for the last 16 hours of incubation.

Tumor growth inhibition assay

10 athymic nude mice were injected subcutaneously with the suspension of DU145 cells (0.1 ml of growth medium with $5x10^5$ cells per site, 4 sites per mouse). 5 mice were left untreated while 5 mice were injected intravenously once a day for 6 days with 0.15 ml of nondiluted conditioned media prepared as described above. Mice were examined weekly for tumor growth; the appearance of tumors detectable by palpation is indicated in Figure 1.

Northern blot hybridization and semi-quantitative RT-PCR

The assays were performed according to standard and previously described protocols in the references cited and incorporated by reference herein.

Immunohistochemical detection of p53 and Western blot analysis of p53 expression and in situ beta-galactosidase assays

These assays were performed as described (Komarova et al., 1997).

Sequence analysis

DNA and mRNA sequences were retrieved from GENBANK Database and searched for p53 binding sites within the range of ±2 kb relative to the trascription start point. One of two additional mismatches and 1-7 bp gaps between half-sites were allowed. Weakened consensus RVNCHWGYYBN$_{1-8}$RVCWHGYYB was used to allow for naturally occurring mutations in known p53 binding sites. Resulting sequences were accepted if they had no more than 3-4 mismatches in total from the previously defined consensus RRRCWWGYYYN$_{0-13}$ RRRCWWGYYY (El-Deiry et al., 1992)and no more than 1 mismatch was allowed inside CWWG core sequences per half-site dimer.

RESULTS

Identification of new p53-responsive genes

The spectra of the p53-responsive genes that alter their expression in response to gamma irradiation in a series of cell systems (source of RNA for hybridization) differing in their origin and p53 status were determined. Specifically, p53-negative fibroblasts MDAH041 and its derivative, TR9-7, which express tetracycline-regulated wild type p53 *cDNA* (Agarwal et al., 1995) were used. The basal level of p53 expression in TR9-7 cells in the presence of tetracycline is similar to that of normal human diploid fibroblasts; accumulation of p53 in these cells is obtained by either gamma irradiation or tetracycline deprivation. In addition to human fibroblasts, mRNA from the spleen, thymus, and liver of wild type or p53-knockout mice (Donehower, et al., 1992; Jacks et al., 1994) either untreated or taken four hours after 10Gy of gamma irradiation. Cells in the thymus and spleen undergo massive p53-dependent apoptosis in response to DNA-damaging treatments, while neither liver cells nor fibroblasts display any evident fast response to DNA damage (Lowe et al., 1993c; Komarova et al., 1997; MacCallum et al., 1996). ^{32}P-labeled complex cDNA probes were synthesized for each cellular mRNA populations and hybridized with the CLONTECH ATLAS cDNA arrays (website www.clontech.com) (human or mouse, depending on the

mRNA origin) each containing 588 cDNAs. Fluorescently labeled cDNA probes from human fibroblasts were hybridized with two microarrays composed of about 20,000 known and unknown human cDNAs (Schena et al., 1995). Differential expression of the genes detected by array hybridization was confirmed by Northern blot-hybridization and/or by semi-quantitative RT-PCR. The differential hybridization results obtained with the above set of cDNAs allowed the identification of mRNA species whose expression was affected by gamma irradiation, and distinguished between p53-dependent and p53-independent genes. The results obtained are illustrated by Fig.2A and summarized in Tables 1 and 2.

Table 1: Mouse p53-responsive genes identified by hybridization to mouse cDNA array

#	Gene	Accession Number	Regulation			Putative p53 DNA-binding sites*
			spleen	Thymus	liver	
1	Cyclin G	Z37110	+	+	+	Okamoto et al., 1994
2	Cyclin B1	X64713	+	(-)	UD	nd
3	Waf-1	U09507	+	UD	UD	El-Deiry et al., 1993
4	p19ink4	U19597	+	NI	UD	nd
5	EI24	U41751	+	+	NI	>+470; >+871
6	Glutathione peroxidase	X84742	-(+)	NI	UD	NF
7	Glutathione S-transferase Pi	U15654	NI	NI	-(+)	-1249; +614
8	Glutathion S-transferase Mu1	J03914(R)	+	NI	NI	+167
9	Bax	L22472	+	+	UD	Miyashita et al., 1995
10	Alpha 1-proteinase inhibitor 2	X66454(R)	+	UD	NI**	-1153; +1489
11	Serine proteinase inhibitor 2	X16361	+	UD	+	-272
12	Serine proteinase inhibitor 2.4	X69832	+	UD	+(+)	>+566***
13	Nerve growth factor alpha	X01801	+(-)	UD	UD	NF
14	Neuroleukin	M14220	-(+)	NI	-(+)	>+642; >+834***
15	EGF	U69534	NI	NI	+	NF
16	Granulocyte-macrophage CSF receptor	M85078	+	UD	UD	NF***
17	Macrophage CSF-1 receptor (CSF-1)	U63963(H)	NI	NI	+	>+862; >+1540
18	Activating transcription factor 4	X61507	+	UD	UD	NF***
19	c-Jun protooncogene	U60582	UD	+	UD	NF
20	p45 NF-E2-related factor 2	U70474	+	NI	+	-608; -93
21	Cathepsin H	U06119	-(+)	NI	-(+)	>+986; >+1192
22	Alpha-actinin binding protein (Zyxin)	X99063	+	NI	UD	NF***

*position of putative p53 DNA-binding sites (distance from transcription start)
**very high expression level
***only coding sequence available
Abbreviations: + and -, p53-dependent induction and suppression; (+) and (-), p53-independent induction and suppression; NI, noninducible; UD, undetectable; NF, not found; nd, not done.
If no genomic sequences were available, closest homologs from other mammalian species were used: (R) - rat sequence; (H) - human sequence.

Table 2. Selected p53-responsive genes identified by hybridization to human cDNA arrays in human fibroblasts.

#	Gene	Accession #	Regulation	Putative p53 DNA-binding sites*
1	WAF1	U24171	+	El-Deiry et al., 1993
2	Glutathione peroxidase	M83094	+	-742; -211; +1548
3	Glutathione S-transferase mu	M96233 X68677	+	+529; +911
4	Inhibin-β A	U16239	+	+1484; +1743
5	TGF-β2	M87843	+	+1580
6	ID-1	U57645	+	+1538
7	ID-2	M97796	+	NF**
8	Ezrin	X51521	+	>+1791; >+1936**
9	Collagen α1 type IX	X54412	+	NF**
10	Collagen α1 type IV	X12784	+	-1095; +901; +1050
11	Collagen α1 type XIV	Y11709	+	>+871; >+584**
12	Collagen α1 type I	AF017178	+	+84; +595
13	Collage α2 type 1	AF004877	+	-919; +57
14	Microfibril-associated glycoprotein 2 (MAGP2)	U37283	+	NF**
15	Glia derived nexin-1	U33453	+	-1602; -1197
16	Antileukoproteinase 1	X04502	+	NF
17	Thrombospondin 2	W86006	-	Adolph et al., 1995
18	Interstitial collagenase	U78045	-	-569; +443
19	Hyaluronan synthase 2 (HAS2)	U54804	-	>+1596**
20	Stromelysin-1	U43511	-	-1579
21	Cathepsin O ≡ Cath K	U83273	-	>-762; >-501
22	Thrombomodulin	AA057068	-	Nd
23	Tissue factor pathway inhibitor-2	L27624	-	Nd
24	Plasminogen activator inhibitor-2, placental	AA032091	-	Nd
25	Prostaglandin G/H synthase	D28235	-	NF

*position of putative p53 DNA-binding sites (distance from transcription start)
**Only RNA sequences available
#1 - #8, genes identified using CLONTECH Atlas
#9 - #25, genes representing the strongest differentials among known genes in large (QBI) cDNA arrays

The genes identified include a number of known p53 responders (Tables 1 and 2): p21/*waf1*, *cyclin G*, *bax* and several genes encoding enzymes of glutathione metabolism. Only one gene, *cyclin G* (Okamoto et al., 1994), among those tested, was recognized as a p53 responder in all mouse tissues analyzed. p53-dependent upregulation of p21/*waf1* and *bax* was evident in some of the cell types tested but was undetectable in others, possibly due to low basal expression levels. Transcription of the E124 gene (Lehar et al., 1996), was activated by gamma irradiation in all mouse organs tested, but in the liver, this activation was p53 independent.

By comparing gamma-irradiated wild type p53 and p53-deficient human fibroblasts, several genes were found to be strongly repressed in the p53-wild type cells (nos. 18-25 in Table 2 and Figs. 3A and 3B). Since in the experiments, mRNA was isolated shortly after the p53 activation, only those negatively regulated genes that were repressed prior to the p53 activation could reliably be detected. In fact, mRNA expression of such genes as stromelysin-1 (Figs. 3A and 3B), Thrombomodulin or prostaglandin G/H synthase was deeply repressed in TR9-7 cells under normal conditions of growth (Table 2).

The largest group of p53 responders includes genes that are upregulated after gamma irradiation on p53 wild type but not on p53-null background (*p21/waf1*, *cyclin G*, *TGF-β2*, etc). Another large group involves genes which basal expression levels was higher in p53-null cells than in p53 wild type cells. They were upregulated in p53 wild type cells and, in some cases, in p53-null cells after gamma irradiation (*neuroleukin*, *ID-1*, *ID-3*, see Figs. 3A and 3B). Another group of genes are subjects of complex regulation (Figs. 2A and 2B). For example, cyclin B1 that is downregulated after gamma irradiation through the known mechanism involving mRNA degradation (Maity et al., 1995) was upregulated by p53 induced in TR9-7 cells by tetracyclin deprivation (Figs. 3A and 3B). The combined effects of the p53-dependent and p53-

independent mechanisms of cyclin B1 explains why the radiation dependent decrease in cyclin B1 mRNA was more pronounced in the p53-null than in the p53-wild type background (Figs. 3A and 3B).

Interestingly, most of the arrayed genes that change expression after gamma irradiation were found to be p53 dependent. For example, among 26 radiation-responsive genes of the spleen, 19 were p53 dependent, confirming a unique role of p53 in radiation response. Different cell types vary dramatically in their content of p53-responsive genes. Only 8 of 19 p53-responsive genes in the spleen are found to respond to p53 in other cell types analyzed. Remarkably, thymus and spleen, which both are highly sensitive to p53-dependent apoptosis, exhibit almost non-overlapping sets of p53-dependent gene expression (Table 1). The tissue specificity of p53 responsiveness of some of the identified genes seems to simply reflect the differences in the basal levels of their expression among tissues. For example, p53-induced upregulation of *p19/ink4/arf* was evident in the spleen, where its basal level is low, and could not be detected in the thymus where its expression was high. *p19/ink4/arf* expression in the liver was below the sensitivity of hybridization. However, several genes (EGF, CSF-1, zycin, GST-μ1) with similar basal levels of expression in different tissues showed tissue specific response to p53, suggesting that p53 pathway can be modulated by unknown tissue specific factors. Our observation on tissue specificity of the majority of p53 responders suggests that some unknown factors target p53 to different groups of promoters.

p53-dependent cellular secretion of growth inhibitors *in vitro*

Newly identified p53 responders can be organized into several groups according to their responses to p53 and their functional activities. One of the major subgroups includes several genes encoding enzymes of glutathione metabolism, reflecting the recent discovery of p53 involvement in the regulation of reactive oxygen (Hermeking et al., 1997).

Other subgroups involve transcription regulators (activating transcription factor 4, c-Jun protooncogene, p45 NF-E2 related factor 2 and ID-1, ID-2), growth factors and their receptors (nerve growth factor alpha; neuroleukin; EGF; granulocyte-macrophage CSR receptor; macrophage CSF-1 receptor(CSF-1)), and extracellular matrix proteins (collagen α1 type IX; collagen α1 type IV; collagen α1 type XIV; collagen α1 type I; collagen α2 typeI). Some of the genes found are subjects of complex regulation. For example, basal levels of neuroleukin and cathepsin H mRNAs were significantly decreased in p53 wild type cells and were upregulated after gamma irradiation in p53-independent manner. Expression of serine proteinase inhibitor 2 is induced by radiation in both p53 wild type and p53-null splenocytes, but induction was significantly stronger with p53 (Table 1, Fig.2A).

Some genes that were upregulated in p53-dependent manner after gamma irradiation in spleen, liver, and fibroblasts encode secreted proteins with a known growth inhibitory function. Different secreted inhibitors were activated in different cell types. They include TGF-β2, inhibin-β (in fibroblasts), and a variety of serine protease inhibitors (*spleen, liver and fibroblasts*; alpha 1 - proteinase inhibitor 2; serine proteinase inhibitor 2; serine proteinase inhibitor 2,4 and glia derived nexin-1; antileukoproteinase 1; thrombospondin 2). Insulin-like growth factor-binding protein 3 (IGBP3) defined earlier as a p53 responder (Buckbinder et al., 1995) also belongs to this group, although its p53-dependent upregulation was visible only in human fibroblasts. The presence of a large number of secreted inhibitors among p53-responsive genes suggests that p53-dependent growth control may not be limited only to suppression of damaged cell proliferation by internal mechanisms, but may also involve induction and release of growth inhibitory factors affecting the surrounding cells (bystander effect). To test this hypothesis, the effect of conditioned media from cells differing in their p53 status before and after p53 activation was compared. As a source of

conditioned media, used several experimental cell systems, all representing isogenic cell lines differing in p53 status or activity was used. Specifically, (i) p53-null human fibroblasts MDAHO41 were compared with their derivatives TR9-7, expressing tetracycline-regulated p53 (Agarwal et al., 1995); (ii) ConA cells (Komarova et al., 1997) were compared with their variant in which p53 function was suppressed by transduction of a strong anti-p53 genetic suppressor element GSE-56 (Ossovskaya et al., 1996); (iii) wild type mouse embryo fibroblasts (MEF) transformed with an E1a and Ha-ras (line C817; Lowe et al., 1993a) were compared with C8 cells transduced with GSE-56 or bcl-2 and with similar fibroblasts from p53-knockout mice; line at (Lowe et al., 1993a; Nikiforov et al., 1997). All the above cells were gamma irradiated (10 Gy) to induce p53. Conditioned medium was collected 8 hours after irradiation and compared with that from control non-irradiated cells in a growth inhibition assays. Along with gamma irradiation, tetracycline deprivation was used to activate p53 in TR9-7 cells.

Several cell types were used as targets in growth-inhibition assays. They include three prostate carcinoma cell lines, PC3, DU145, and LNCAP and three nearly normal cells: human and mouse diploid fibroblasts and nontransformed murine mammary gland epithelial cells NmuMG. Similar results were obtained with all cell lines, and we chose DU145 cells as reference target cells for further analysis because they are known to be the most resistant to conventional anticancer treatments among the tested cell lines (Rokhlin et al., 1997).

The results of ^{3}H-thymidine incorporation assays done on DU145 cells are shown in Figs. 4A-4C. DU145 cells were chosen for the demonstration of the effect since they are resistant to many known negative regulatory stimuli, including TGF-β, TNF-α, interferon-γ and FAS ligand; these cells are p53-deficient and do not undergo apoptosis in response to treatment with radiation or DNA-damaging chemo therapeutic drugs (Rokhlin et al., 1997). Conditioned medium from untreated p53 wild type cells did not affect the growth of

target cells. The medium from p53-negative cells had no detectable growth inhibitory effects, regardless of whether or not they were irradiated. In all three cell systems, growth inhibition of target cells was evident only with the medium collected from p53 wild type cells after p53 activation. Growth inhibition was evident in all target cell types tested, although the scale of the effect varied among different cells (Fig. 5C). The strength of the growth inhibitory effect correlated with the concentration of conditioned media and could be enriched by ultrafiltration through Amicon filters with the cutoff of 10kd (Fig. 5C).

It is important to note that radiation did not induce apoptosis in human fibroblasts or ConA cells within the time of the experiment, as judged by a DNA fragmentation assay (data not shown), ruling out the possibility that the growth-suppressive effect of the medium reflects cell degeneration. Moreover, *bcl*-2 overexpression did not prevent C8 cells from secreting growth inhibitory factors into the medium, although it blocked apoptosis associated with p53 activation in C8 cells (Nikiforov et al., 1997) (Fig. 4A).

p53-dependent secretion of growth inhibitors *ex vivo* and *in vivo*

Whether p53-dependent secretion of growth inhibitors occurred in primary cell cultures and *in vivo* in the organism in response to gamma irradiation was also assessed. Spleen and thymus were chosen for these experiments because they both respond to gamma irradiation by activation of massive p53-dependent apoptosis but differ dramatically in contents of p53-responsive genes (Table 1). Short-term cultures of splenocytes and thymocytes from untreated and gamma-irradiated p53-wild type and p53-null mice were prepared and the growth inhibitory effect of conditioned medium at different times after treatment was tested. Similarly, urine samples from wild type and p53-knockout C57BL mice were analyzed before and

7 hours after being subjected to 10 Gy of gamma radiation. The results of representative experiments summarized in Figs. 5A and 5B demonstrate the growth-suppressive effect detected both *ex vivo* in conditioned media from splenocytes and *in vivo* in urine of irradiated animals. In both cases, the effect was p53-dependent (Figs. 5A and 5B). Conditioned media from short-term cultures of irradiated thymocytes did not display any detectable growth suppressive activity (data not shown); consistently, no secreted proteins were found among the products of a few p53-responsive genes of thymus (see Table 1). At the highest concentrations, the conditioned media from irradiated splenocytes induced apoptosis in target tumor cells (Fig. 1). Remarkably, the growth suppressive effects of conditioned medium and urine were more pronounced in the three tumor cell lines tested than in three types of normal cells, including mouse and human diploid fibroblasts and mouse mammary gland epithelial cells NmuMG (Fig. 5C).

p53-dependent bystander effect can potentiate the efficacy of anti-cancer drugs

The p53-dependent secretion of growth inhibitors raises the possibility that chemo- and radiation therapy may act not only by causing direct damage to tumor cells, but also by inducing "export" of anti-proliferative stimuli and the release of growth inhibitors by normal cells in response to stress, thereby affecting growth of their neighbors. To reveal this putative indirect action of chemotherapy, the effect of a chemotherapeutic drug on drug-resistant tumor cells, co-cultivated with nonmalignant fibroblasts, either p53-deficient fibroblasts (MDAHO41) or with their derivatives expressing wild type p53 (TR9-7), was compared. To distinguish between the direct and indirect effects of chemotherapeutic drug treatment on tumor cells, the multidrug-resistant tumor cell line KB8-5-11, which is resistant to adriamycin due to the overexpression of P-glycoprotein (P-gp) (Roninson et al., 1986), was used. KB8-5-11 cells were plated

on sparse monolayers of either p53-deficient (MDAHO41) or p53 wild type (TR9-7) human fibroblasts (1000 KB8-5-11 cells per plate). Such an experimental design imitated tumor growth on p53-deficient or p53-wild type stroma. Once fibroblasts formed a complete monolayer and KB8-5-11 cells formed small colonies, 50 ng/ml of adriamycin was added to the plates. This drug concentration led to complete growth arrest (but not apoptosis) in human fibroblasts (data not shown) and did not affect growth of KB8-5-11 cells plated either alone or on the layer of p53-deficient fibroblasts (Figs. 6A-6D). However, most KB8-5-11 colonies growing on the monolayer of fibroblasts with wild type p53 degraded, displaying morphological characteristics of apoptosis (Figs. 6A-6D). This observation demonstrates that the killing effect of chemotherapeutic drugs on cancer cells can be determined in part by the p53 status of surrounding normal cells - apparently through the p53-dependent induction of secreted growth inhibitors. Similar experiments were performed using spontaneously transformed Balb 3T3 cells co-cultivated with on MDAHO41 and TR9-7 cells. In these experiments, the induction of p53 expression was achieved in TR9-7 cells by tetracycline deprivation. As in the previous case, the growth of colonies of transformed cells was significantly reduced on the TR9-7 background (data not shown). These results demonstrate that activation of p53 in normal cells leads to growth inhibition of co-cultivated tumor cells apparently through bystander effect by surrounding nonmalignant cells caused by the release of growth inhibitors. This observation shows that the p53-dependent response of normal tissues to anticancer treatments can facilitate the efficacy of chemo- and radiation therapy.

The p53-dependent secretion of growth inhibitors with potential anticancer activity detected both in conditioned medium and in body fluids indicates a novel growth regulatory function of p53: stress-dependent cellular export of growth inhibitory stimuli. This function possibly determines the "bystander effect" associated with p53 gene therapy (Qazilbash et al., 1997) .

The list of identified growth inhibitors demonstrates their involvement in many well-known mechanisms of negative growth relation that have never before been associated with p53. Even a single group of p53-dependent secreted proteins serine protease inhibitors, can inhibit cell proliferation through numerous signaling pathways (reviewed by Feinstein et al.). p53 can serve as a transcriptional suppressor (Mack et al., 1993), and several genes that are negatively regulated by p53 were found in this study (see Tables 1 and 2). Some of the gene products of these genes have known functions that could contribute to the "exported" growth inhibitory effect of p53. Thus, deep suppression of hyaluronan synthase 2 (HAS2) is expected to cause a detrimental effect on cell proliferation by leading to a drop in the synthesis of hyaluronan which is the involved in a variety of growth control pathways (Rooney et al., 1995). Remarkably, the observed growth suppression effect of p53-dependent secreted factors does not depend on the origin (mouse or human) and p53 status of the target cells: DU145 and PC3 cells are p53-deficient, while the rest of the target cells tested have wild type p53.

Some of the p53-dependent factors have profound anticancer activity and are already used as supplements to chemotherapeutic treatments. These include members of the TGF-β family and numerous serine protease inhibitors and their chemical functional analogs, which are emerging as potential tools to treat cancer (DeClerck et al., 1994).

The p53-dependent modulation of gene expression in gamma-irradiated fibroblasts (Table 2) clearly reflects morphological and physiological alterations in irradiated tissues associated with growth attenuation of tumors transplanted in gamma irradiated organs (O'Brien et al., 1969) known as the tumor bed effort (TBE). Thus, induction of various collagen species and suppression of collagen-degrading proteinases (stromelysin-1, interstitial collagenase and cathepsin K) correlates with deposition of collagen by connective tissue in coarse bundles following irradiation.

Deep p53-dependent suppression of HAS2 and COX2 genes, determining production of potent angiogenic factors hyaluronan and prostaglandin E (Rooney et al., 1995; Gullino et al., 1995), along with the previously found activation of thrombospondin 1 (Dameron et al., 1994), correlates with the suppression of angiogenesis in irradiated tissues (O'Brien et al., 1969). These observations indicate that TBE is a p53-dependent phenomenon.

So far, p53-dependent secretion of biologically active factors was known to be involved in control of angiogenesis (Dameron et al., 1994). The data show that this mechanism of stress response involves numerous factors and affects many different cell types, allowing one to revise the role of p53 in the stress response *in vivo* and in anticancer therapy by adding a new growth regulatory function associated with this tumor suppressor. The expansion of this study to other tissues and larger cDNA arrays is a straightforward way to identify other p53-responsive secreted growth inhibitors that would further our knowledge of the mechanisms of p53-regulated stress response and lead to identification of new biologically active molecules with potential therapeutic applications.

Morphological and physiological alterations of normal tissues associated with aging may well be another phenomenon related to the newly determined function of p53. Senescent cells that accumulate in tissues over time (Dimri et al., 1995) are known to maintain very high levels of p53-dependent transcription (Atadja et al., 1995). p53-dependent secretion of growth inhibitors by senescent cells, accumulating in aging tissue, may affect proliferating cells and lead to a gradual decrease in overall proliferation capacity of tissues associated with age. Identification of p53-dependent growth inhibitors allows this hypothesis to be tested directly.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters,

concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by references.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be

understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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What Is Claimed Is:

1. A method for preparing p53-dependent growth inhibitory factors, comprising the steps of:

culturing human cells capable of expressing p53 tumor suppressor protein in a nutrient medium;

inducing expression and secretion of p53-dependent growth inhibitory factors by the cultured human cells into the nutrient medium to form a conditioned medium; and

recovering the p53-dependent growth inhibitory factors from the conditioned medium to provide a preparation of the p53-dependent growth inhibitory factors.

2. The method according to claim 1, wherein the nutrient medium for culturing human cells is a serum-free medium.

3. The method according to claim 1, wherein the cultured human cells are fibroblasts.

4. The method according to claim 1, wherein the cultured human cells are a mixture of cell types capable of expressing p53 tumor suppressor protein and capable of being induced to express and secrete p53-dependent growth inhibitory factors.

5. The method according to claim 1, wherein the p53-dependent growth inhibitory factors expressed and secreted by the cultured human cells are selected from at least one of the group consisting of TGF-beta 2, inhibin-beta, and serine protease inhibitors.

6. A pharmaceutical composition for reducing unwanted or abnormal cell proliferation, comprising a preparation of p53-dependent growth inhibitory factors prepared according to claim 1.

7. The pharmaceutical composition according to claim 6, further comprising a pharmaceutically acceptable carrier, excipient, diluent or auxiliary agent.

8. A method for reducing unwanted or abnormal cell proliferation, comprising the step of administering an effective amount of the pharmaceutical composition according

to claim 6 to a subject in need thereof.

9. The method according to claim 8, wherein the cell proliferation is cancer.

10. The method according to claim 9, further comprising administering a chemotherapeutic compound to the subject or irradiating the subject with gamma radiation.

11. A method for preventing or delaying cell senescence, comprising the step of administering to a subject in need thereof an effective amount of a pharmaceutical composition comprising an agent which attenuates the secretion of or neutralizes the growth suppressive effects of one or more p53-dependent growth inhibitory factors.

FIG. 1

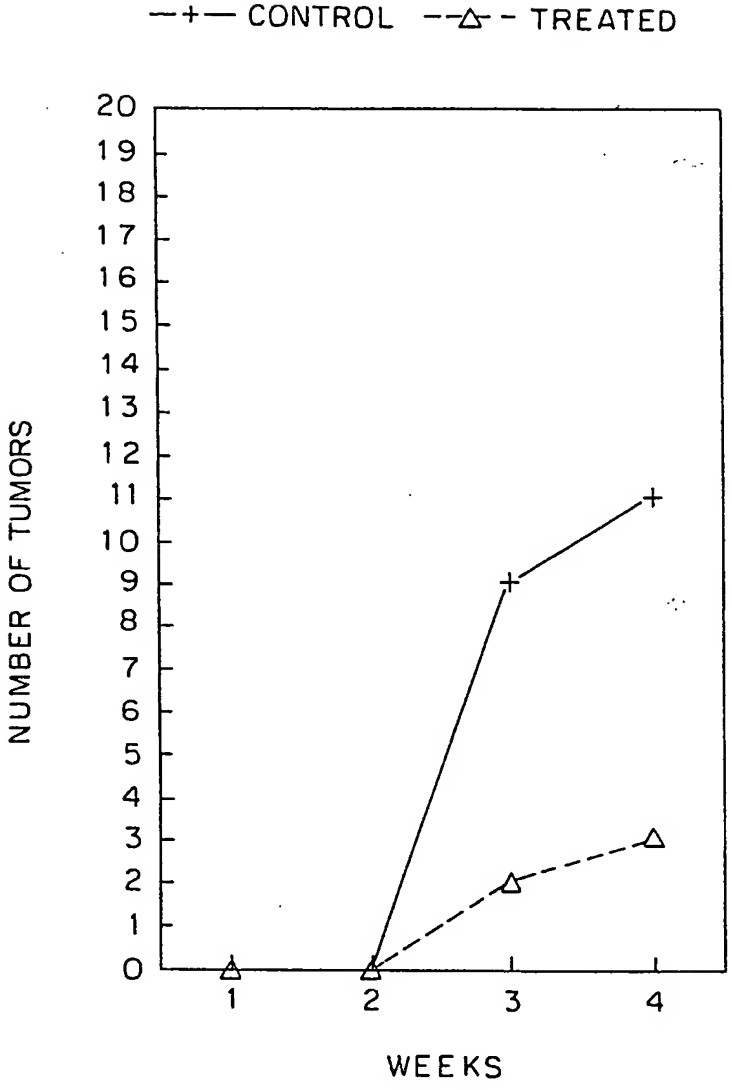

—+— CONTROL --△-- TREATED
NUMBER OF TUMORS
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
WEEKS

FIG. 2A

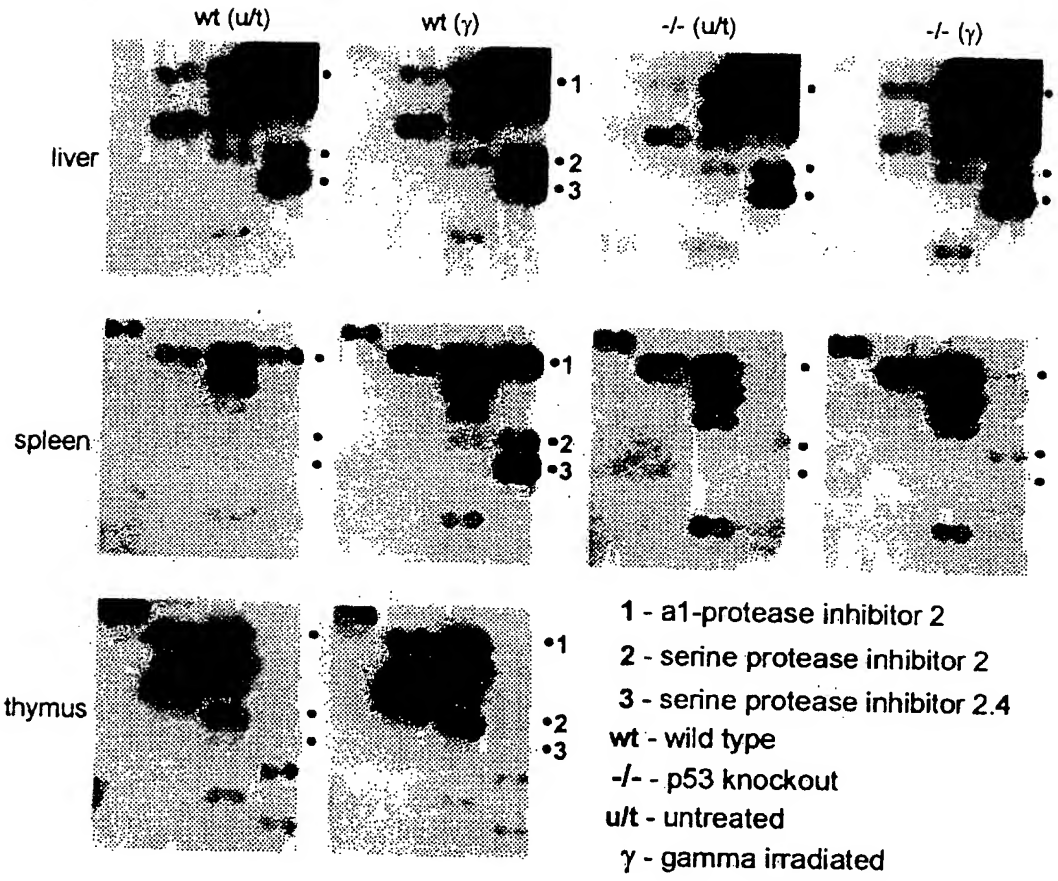

wt (u/t)
wt (γ)
-/- (u/t)
-/- (γ)
liver
spleen
thymus
•1
•2
•3
1 - a1-protease inhibitor 2
2 - serine protease inhibitor 2
3 - serine protease inhibitor 2.4
wt - wild type
-/- - p53 knockout
u/t - untreated
γ - gamma irradiated

FIG. 2B

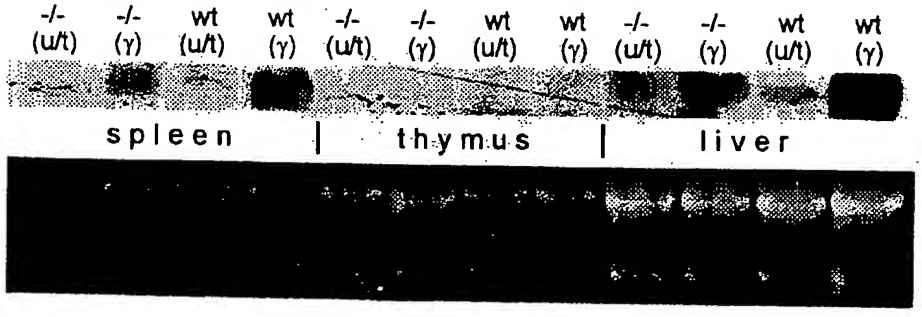

-/- (u/t)
-/- (γ)
wt (u/t)
wt (γ)
-/- (u/t)
-/- (γ)
wt (u/t)
wt (γ)
-/- (u/t)
-/- (γ)
wt (u/t)
wt (γ)
spleen
thymus
liver

FIG. 3A

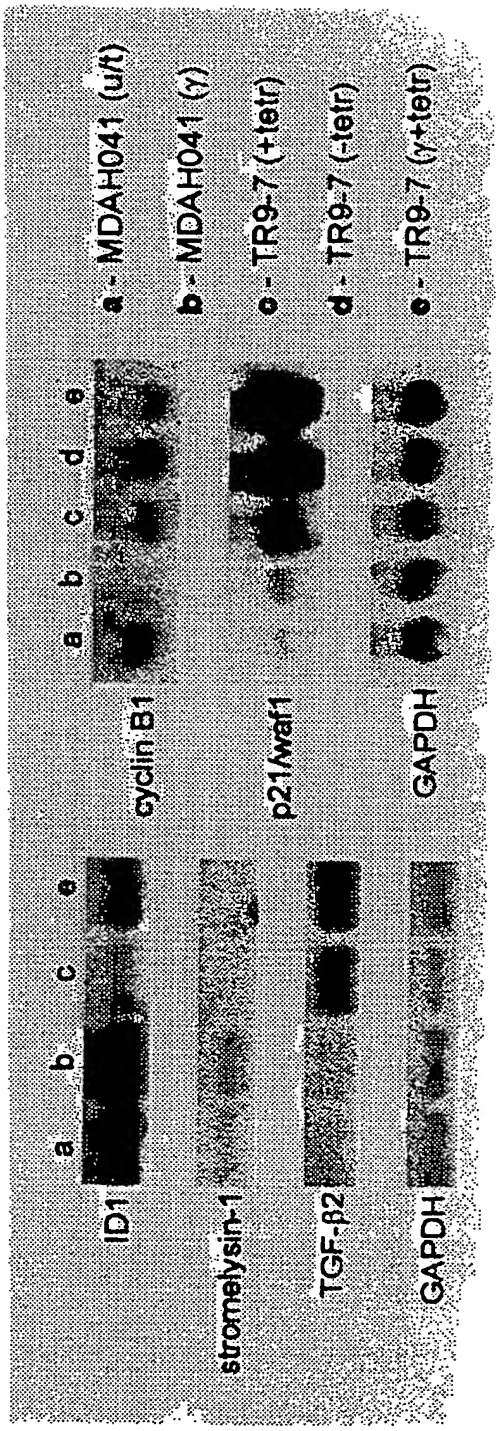

a
b
c
e
ID1
stromelysin-1
TGF-β2
GAPDH

FIG. 3B

a - MDAH041 (u/t)
b - MDAH041 (γ)
c - TR9-7 (+tetr)
d - TR9-7 (-tetr)
e - TR9-7 (γ+tetr)
a
b
c
d
e
cyclin B1
p21/waf1
GAPDH

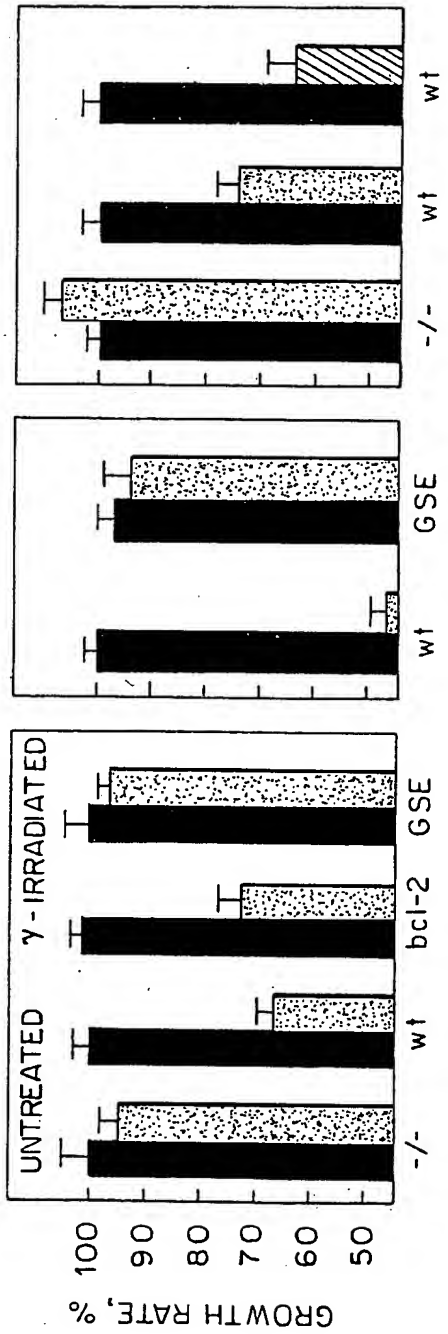


FIG. 4A FIG. 4B FIG. 4C

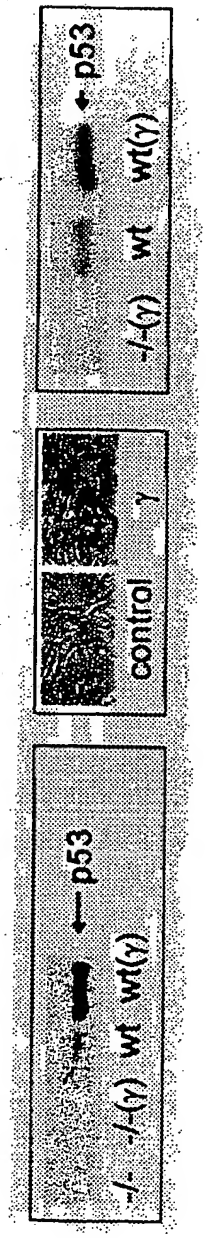


FIG. 4D FIG. 4E FIG. 4F

FIG. 6A

FIG. 6B

FIG. 6C

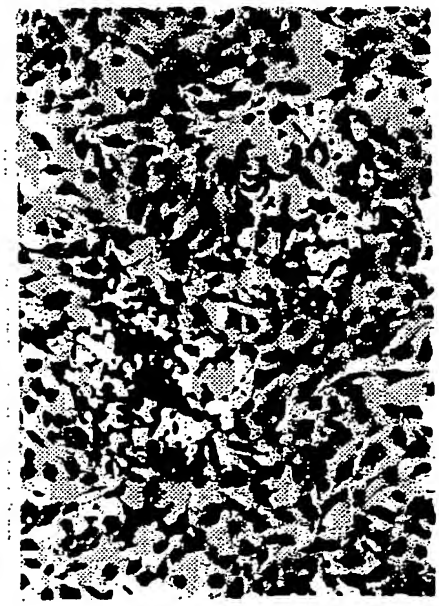


FIG. 6D

FIG.5A

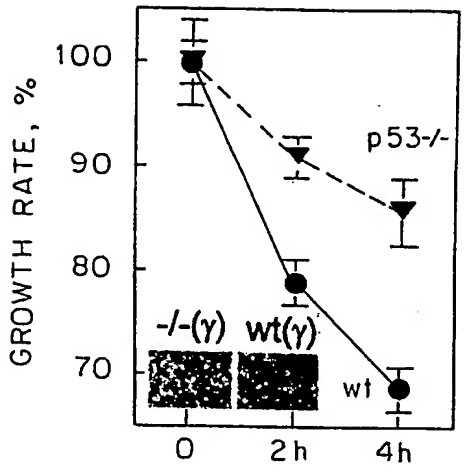


FIG.5B

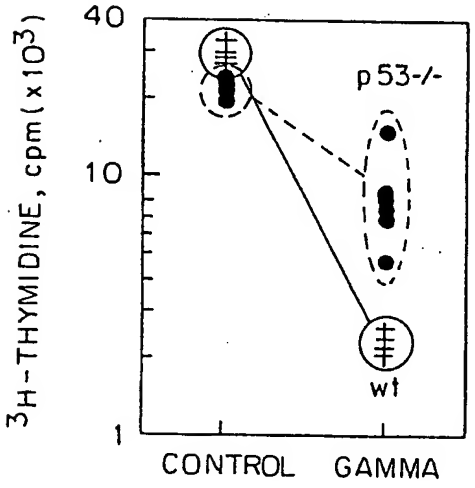


FIG.5C

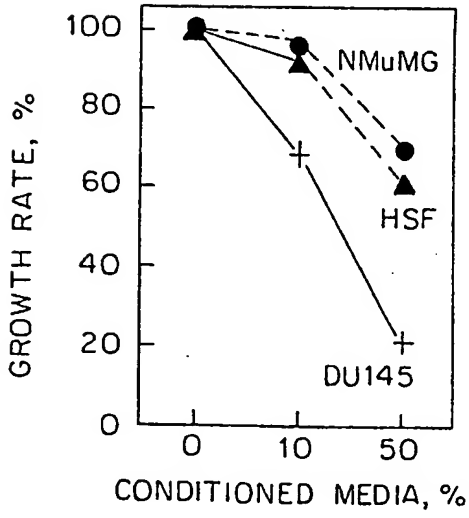
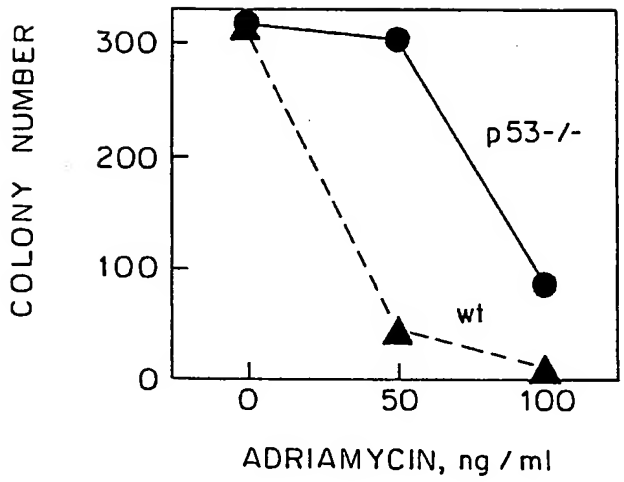


FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03777

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 37/18; C12N 5/06; A61K 38/60; C07K 1/00
US CL : 514/2; 435/325; 530/344, 361

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 435/325; 530/344, 361

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Database SCISEARCH on STN. TANG et al. 'Expression of Transforming Growth-Factor-Beta (TGF-BETA) Isoforms and TGF-Beta Type II Receptor Messenger-Ribonucleic-Acid and protein, and the Effect of TGF-Beta-S on Endometrial Stromal Cell-Growth and Protein Degradation In-Vitro'. Endocrinology. July 1994, Vol. 135, No. 1, pages 450-459, see Abstract.	1,4,5,6 ------- 2,3,7
X -- Y	Database CAPLUS on STN. BALE et al. 'Regulation of Insulin-Like Growth Factor Binding Protein-3 Messenger Ribonucleic Acid expression by Insulin-Like Growth Factor I'. Endocrinology. 1992, Vol 131, No. 2, pages 608-614, see Abstract.	1,3,6 ----- 2,7
A,P	Database EMBASE on STN. KOMAROVA et al. 'Stress-Induced Secretion of Growth Inhibitors: A Novel Tumor Suppressor Function of p53'. Oncogene. 03 September 1998, Vol. 17, No. 9, pages 1089-1096, see Abstract	1-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
14 MAY 1999

Date of mailing of the international search report
28 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03777

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03777

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, CANCERLIT, CAPLUS, DDFU, DRUGU, EMBASE, MEDLINE, SCISEARCH, TOXLINE, TOXLIT, USPATFULL
search terms:culture, secrete, fibroblast, human, tgf-beta 2, cancer, tumor, treat therapy, senescence, neutralize

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-10, drawn to a pharmaceutical composition for reducing unwanted or abnormal cell proliferation, that is a p53 growth inhibitory factor, a method of making and a method of using said composition.

Group II, claim(s) 11, drawn to a method of preventing or delaying cell senescence comprising an inhibitor of the composition of Group I.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is considered to be a p53 growth inhibitory factor.

The special technical feature of Group II is considered to be an inhibitor of p53 growth inhibitory factor.

Accordingly, Groups I-II are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.